

CAROTENOID PIGMENTS IN THE COXAL GLAND OF *LIMULUS*

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Packard (1875) was the first to call attention to an organ in *Limulus polyphemus* which he stated (p. 511) was "conspicuous from its bright red color." Lankester (1882, p. 95) in his studies on this tissue referred to it as the "brick-red gland." He noted the structural resemblance of this tissue to the colorless coxal glands of *Scorpio*, which led to the development of his much-debated thesis (cf., Versluys and Demoll, 1922) that *Limulus* was to be classed as an arachnid. Following these reports, a number of anatomical studies by Gal-land (1885) and Patten and Hazen (1900) appeared which provided evidence in support of the view originally expressed by Packard (1875, p. 513) that the red coxal gland of *Limulus* was "renal in its nature." More recent studies with the electron microscope by Karnovsky and Briggs (Harvard Medical School, personal communication) extend the evidence that this tissue has a morphological structure resembling that of a nephridium. Though Packard, over a hundred years ago, described this tissue as conspicuous for its bright red color, more conspicuous today is the apparent absence of any reports in the literature on the chemical identity of its coloring matter. The present investigation was therefore undertaken in an attempt to supply this missing information. Evidence is presented that the red color of the coxal gland of *Limulus* is due to its high content of carotenoids.

MATERIALS AND METHODS

The coxal gland of *Limulus* is a bilateral organ with a total of eight lobes which lie embedded in the muscles around the base of the second, third, fourth, and fifth legs. In this study large adult animals were employed (carapace width 15-28 cm), and the tissue from all the lobes was trimmed free of muscle and pooled. No attempt was made to effect a quantitative recovery of the tissue, and the total amount obtained varied from 250 to 1350 mg, depending upon the size of the animal. The tissue was ground in an all-glass homogenizer with absolute methanol, the mixture centrifuged, and the supernatant solvent collected. Extraction was repeated until no further pigment was removed, and the nearly colorless residue was discarded. Water was added to the methanol solution to bring its concentration to 90% and extracted with petroleum ether (bp, 30-60° C) until the extracts were colorless. The petroleum ether solution was washed with water to remove methanol and subjected to column chromatography. Columns of an aged aluminum oxide preparation were found most useful for this purpose using petroleum ether with increasing concentrations of acetone to separate and elute the carotenoids. The purity of the various fractions yielded by this procedure was checked and R_F values obtained by thin layer chromatography on silica gel plates using either a 20% acetone-80% hexane or a 20% acetone-80% petroleum ether as a developing solvent. Spectrophotometric determinations were made with a Beckman DU

spectrophotometer on which the wavelength setting was checked using the 550 nm band of reduced cytochrome c as a reference point. Organic solvents employed were analytical or spectrophotometric grade. During all procedures an effort was made to exclude light and to keep solutions under a nitrogen atmosphere.

RESULTS

Initial experiments revealed that no pigment could be extracted by homogenization of the tissue with water or 0.5 M NaCl solutions. Examination of the intact tissue or aqueous homogenates with a hand spectroscope revealed no bands characteristic of the hemochromogens, either before or after the addition of the reducing agent dithionite. The bulk of the pigment could be readily extracted with either acetone or methanol. Spectroscopic examination of such extracts revealed a spectrum characteristic of the carotenoids with a peak at 450–453 nm and a shoulder at 475 nm. Dilution with water of a methanol solution to 90% permitted the extraction of over 90% of the pigment by petroleum ether. The pigment remaining in the methanol phase displayed the spectrum of carotenoids and could be extracted into petroleum ether or chloroform upon further dilution of the methanol with water. Further characterization of this pigment was not attempted.

Total carotenoid content

An estimate of the total carotenoid content of the coxal gland was made by measuring the optical density of an extract of the gland at its absorption maximum, usually 450 nm. For this purpose a value of 2500 for $E_{1\%}^{1\text{cm}}$ was employed. Measurements were made either on the original methanol extract or most often on the petroleum ether extract of the diluted methanol solution. The total carotenoid content obtained in this manner ranged from 25 to 226 μg per gram of wet tissue. The corresponding average value for glands from 21 animals (11 females, 10 males) was 111 μg . Glands which had a brick-red color to the eye contained the highest content of carotenoids. In many cases the intact gland has a yellow color to the eye but reveals a red color upon cross section. In one experiment the exterior yellow portion and the interior redder portion were dissected free from one another. The carotenoid content of the pale exterior portion was 62 μg and that of the interior redder portion 210 μg per gram wet weight. There is some indication that glands with the highest carotenoid content are found in those animals fresh from their native habitat, although this requires further documentation.

Separation and partial identification of carotenoids

The multiple nature of the carotenoids in the coxal gland is clearly revealed by column or thin layer chromatography of a petroleum ether extract. Seven to eight components are clearly recognizable by these procedures. An example of the results to be obtained is illustrated by the data presented in Table I. In this experiment the glands from three animals were used, and 91% of the carotenoids were removed by petroleum ether from the 90% methanol extract. A portion of the petroleum ether extract was added to a column of aluminum oxide, 9×1 cm, and developed with petroleum ether containing increasing amounts of acetone, as indicated in Table I. The fractions containing acetone were evaporated to dry-

TABLE I

Relative abundance and chromatographic and spectral characteristics of coxal gland carotenoids.

Fraction	Eluent	Absorption peaks nm	R _F	Percentage of total
1	Petroleum ether	449,475	0.97	8.6
2	1% Acetone*	455	0.55	28.8
3	2% "	455	0.48	7.0
4	5% "	449,475	0.46	3.4
5	5% "	447,474	0.32	12.3
6	10% "	450	0.26	5.5
7	20% "	447	0.22	5.2
				70.8

* Percentage volume of acetone in a petroleum ether-acetone mixture.

ness in the dark under a stream of nitrogen and the residue dissolved in petroleum ether for spectrophotometric and R_F determinations. The percentage of the total recovered in each fraction was determined from the volume and optical density at 450 nm of the fraction, as compared to measurements made on the original petroleum ether extract. The R_F values were determined on silica gel plates developed with a 20% acetone-80% hexane mixture.

As judged by the results of thin layer chromatography, a fairly clean separation of components was obtained in this experiment and nearly all fractions appeared to contain a single component. Additional information and some of the conclusions that may be drawn as to the nature of some of the various components are as follows.

Fraction 1. The absorption spectrum and R_F value of this component are identical with those obtained on an authentic sample of β -carotene, and it would appear that this component is β -carotene.

Fraction 2. Its absorption spectrum is broad and resembles that of a keto-carotenoid such as echinenone. In 95% ethanol, this component displays a broad absorption band centered at 465 nm. Upon reduction with borohydride, two peaks at 452.5 and 480 nm appear. In petroleum ether, the reduced compound exhibits peaks at 447.5 and 475 nm, and its R_F is 0.40. This major component thus appears to be a keto-carotenoid.

Fraction 3. In 95% ethanol the component in this fraction has a broad absorption band centered at 470-475 nm. Upon reduction with borohydride, two peaks appear at 451 and 480 nm. In petroleum ether, the reduced compound shows two peaks at 449 and 475 nm, and its R_F is 0.38. This component thus appears to be another keto-carotenoid, not unlike that in fraction 2.

Fraction 4. The material in this fraction, unlike the others, does not yield a sharp R_F value, and the value given in the table represents the average of an R_F spread from 0.40 to 0.52. There is thus a possibility that more than one component is present in this fraction.

Fraction 5. The absorption spectrum of this fraction very closely resembles that of the reduced form of the component in fraction 2 suggesting that it contains

a hydroxycarotenoid. No change in spectrum occurred upon treatment of its ethanolic solution with borohydride.

Fraction 6. The absorption peak at 450 nm of this fraction is unusually distinct with a well-defined shoulder at 475 nm.

Fraction 7. In 95% ethanol, the main absorption of this fraction lies at 450 nm. Upon reduction with borohydride, the position of this absorption band remains unchanged but it is sharpened and increased.

After the collection of the seven fractions listed in Table I, which accounted for 71% of the total material added to the column, there still remained pigment on the column near its top. This material could not be removed by either 100% acetone or methanol. Glacial acetic acid did remove most of this material, but further characterization of it has not proved feasible.

It should be emphasized that although there is some similarity in the results obtained from experiment to experiment, noticeable differences are encountered. For example in another run the material in fraction 1, presumably β -carotene, accounted for about 20% rather than 8% of the total. Also in this experiment, fraction 2 represented about 32% of the total, while the total amount recovered from the column was about 85%. Thus, both the total carotenoids present as well as the composition can vary from animal to animal.

Carotenoids in other tissues of Limulus

A less extensive examination of the carotenoids of three other *Limulus* tissues was made so that some comparison could be made with those found in the coxal gland. The extraction of these tissues and the carotenoid measurements were carried out in the same manner as described for the coxal gland. The values given here for these tissues are based upon the examination of a petroleum ether solution obtained by extraction of the original methanol extract after its dilution to 90% with water. On this basis, the hepatopancreas of four animals yielded total carotenoid contents of 30, 32, 15, and 48 μg per gram wet weight, for an average value of 31 μg . Eggs obtained from two animals yielded total carotenoid contents of 8.6 and 15.5 μg per gram wet weight. Analysis of the total blood clot obtained from two animals gave values for total carotenoids of 1.7 and 3.3 μg per gram wet weight. Expressed in terms of the amount present in 100 ml of whole blood these values are 6.1 and 9.7 μg , respectively. In one experiment the anebocytes of the blood were isolated by the technique of Murer, Levin, and Holme (1975). The total content of carotenoids in these cells was 5.7 μg per ml as packed by centrifugation. Since 100 ml of whole blood yielded 1.35 ml of cells, the corresponding value for 100 ml of whole blood is 7.7 μg .

In the case of each tissue the petroleum ether extracts were subjected to thin layer chromatography. Based upon R_F values observed in these experiments, the mixture of carotenoids in the eggs and anebocytes resembled most closely those present in the coxal glands. The three chief carotenoids present in these tissues appeared to be the same as those found in the coxal gland, namely those listed as fractions 1, 2, and 5 in Table I. The mixture of carotenoids present in the hepatopancreas bore the least resemblance to those in the coxal gland, with β carotene (fraction 1, Table I) being the only component that was clearly identifiable. It may also be noted in the case of the hepatopancreas that as measured by total

absorption at 450 nm, only about 50% of the pigment in the 90% methanol extracts is transferable to petroleum ether. The corresponding value for the other tissues including the coxal gland is close to 90%.

DISCUSSION

The brick-red color of the coxal gland of *Limulus* noted by earlier investigators is undoubtedly to be attributed to its high content of carotenoids. The average carotenoid content of the glands from 21 animals was 111 μg per gram wet weight, while the highest value observed was 226 μg . A table of the carotenoid content of marine invertebrates is given by Goodwin (1954). The highest value to be found in this table is 149.6 μg per gram wet weight for the coelenterate, *Metridium senile* (red). Examination of three other tissues of *Limulus* (eggs, blood, and hepatopancreas) revealed that the highest carotenoid content of these three tissues was in the hepatopancreas, which had an average value of 31 μg per gram wet weight. The concentration of carotenoids in the coxal gland is thus much greater than in any of the other tissues that were examined.

It is generally agreed that animals are not capable of the *de novo* synthesis of carotenoids. Their occurrence in animals is therefore to be attributed to dietary intake and the ultimate source is to be traced back to plants in the food chain. Thus, the wide variation in the concentration of carotenoids in the coxal gland reported here, 25–226 μg per gram wet weight, may well represent the previous dietary history of the animals. The specimens of *Limulus* used in this study were not only collected at different seasons of the year and locations, but some were also held unfed in captivity for varying and unknown lengths of time. No correlation between the sex of the animal and the content of carotenoids in the coxal gland was discernible.

The reason for the accumulation of carotenoids in the coxal gland is not clear. Since what evidence is available suggests that this tissue is renal in nature, one premise that may be drawn is that during its excretion of waste products the carotenoids are retained by this tissue for some unknown reason. The possibility that these pigments play some role in the function of the gland can not be ruled out at this time. Indeed, any consideration of this possibility must await a fuller understanding of the functions of the coxal gland. Most of the earlier studies are morphological in nature, and very few reports are to be found on the excretions of this tissue. Tower (1895, p. 472) in his description of the external opening of the coxal gland wrote, "I have many times noticed a white transparent fluid oozing to the exterior through the external openings. Chemical analyses which are now being carried on indicate that the glands are of an active excretory nature." A search of the literature has failed to reveal any subsequent publication of Tower's chemical analyses. More recently, Mangum, Booth, DeFur, Heckel, Oglesby, and Polites (1976) have reported on Na^+ , Cl^- , NH_4^+ concentrations, pH values and total solids in excretions of the coxal gland of *Limulus*. Their data indicate that the concentration of sodium and chloride ions and the total osmolality of the urine and blood are similar for *Limulus* inhabiting waters with the salinity of normal sea water. Concentrations of NH_4^+ in the urine are some eight times higher than in the blood, and the urine is more acid than the blood. Subjecting *Limulus* to water of low salinities results in a lowering of the osmolality of both blood and

urine. Even though rates of urine secretion are not given, these results suggest that the coxal gland is not required to expend much energy in its treatment of the inorganic components of the animal.

It seemed logical to conclude that the immediate source of the carotenoids of the coxal gland was to be found in the blood of *Limulus*. Since the blood clot has a yellowish color, attention was focused on it and the amebocytes that it contains, rather than on the clot-free portion containing hemocyanin. Carotenoids were found to be present in both the whole blood clot and the isolated amebocytes though in concentrations which are relatively small when compared to those of the coxal gland. It is of interest that, as revealed by thin layer chromatography, the mixture of carotenoids found in this fraction of the blood bears a closer resemblance to those in the coxal gland than those present in the hepatopancreas. The amebocytes contain granules, and Murer, Levin, and Holme (1975) have presented evidence that these granules contain all the factors required for the coagulation of the blood. According to Lankester (1884, p. 161) the color of the coxal gland "is due to the presence of numerous small red-coloured granules which occur in the layer of gland-epithelium." It would be of interest to know whether these granules or their contents are derived from the granules of the amebocytes.

An abundant source of both the blood and coxal gland carotenoids could be the hepatopancreas, which is a very large organ in *Limulus* and contains on the average 31 μg of carotenoids per gram wet weight. However, the carotenoids of the hepatopancreas display properties which indicate that they are different from those found in the coxal gland or blood. Hence, metabolic conversions of the hepatopancreas carotenoids must occur if they are the source of those present in the blood and coxal gland. If such conversions occur, the site is not indicated by the studies presented here.

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SUMMARY

The coxal gland of *Limulus polyphemus* contains a high content of carotenoids. Values ranging from 25 to 226 μg per gram of wet tissue were found. The corresponding average value for 21 animals was 111 μg . Chromatographic separation reveals the presence of 7 to 8 components. Characterization and partial identification of these components has been made by the determination of R_F values and absorption spectra. In some cases the alterations produced in these properties by borohydride reduction have also been presented. The brick-red color of this tissue reported by earlier investigators would appear to be largely due to its high content of carotenoids.

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